Structural and functional changes in LDL after modification with both 4-hydroxynonenal and malondialdehyde

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Abstract Oxidized LDL is characterized by adduct formation between apolipoprotein (apo)B-100 in the low density lipoprotein (LDL) particles and reactive aldehydes such as 4-hydroxynonenal (HNE) and malondialdehyde (MDA), which are decomposition products of lipid peroxidation. Since LDL isolated from human atherosclerotic lesions was also shown to have interacted with HNE and MDA and to have undergone oxidation, we initiated a study to structurally and functionally characterize LDL modification with both HNE and MDA and to compare these characteristics with those of LDL modified individually with HNE and MDA. Using concentrations of LDL and HNE that alone resulted in extensive particle aggregation, modification at pH 7.4 with HNE plus MDA at 20 mM and above, resulted in the prevention of particle aggregation and less aggregation of apoB-100. Increases in electrophoretic mobility and blockage of exposed lysine residues on apoB were approximately additive for LDL modified at pH 7.4 with 6 mM HNE and MDA up to 20 mM compared to LDL modified individually with HNE or individually with MDA. When ^{125}I-labeled LDL modified at pH 7.4 with HNE and increasing amounts of MDA was incubated with J774 macrophages, the doubly modified LDL showed a linear increase in degradation and in ACAT stimulation. LDL modified with MDA alone showed first a reduction in degradation and ACAT stimulation, due to reduced recognition by the LDL receptor. This was followed by an increase in degradation when further modification with MDA was performed at pH 6.4, but no or little increase in degradation when performed at pH 7.4. This result was consistent with a reduced interaction of MDA with lysine residues in apoB-100 in LDL at pH 7.4 compared to pH 6.4. Even at equal modification with MDA of lysines on apoB in LDL, macrophage degradation was greater for LDL modified at pH 6.4 than at pH 7.4, suggesting additional changes in LDL that enhanced macrophage recognition. LDL modified with both HNE and MDA was recognized by the scavenger receptor, based on cross-competition studies of degradation between labeled and unlabeled forms of LDL modified with both HNE and MDA and with acetyl LDL, as well as competition with fucoidin. Collectively, these studies suggest that the differences in amounts of specific reactive aldehydes formed and/or availabilities for binding to lysine residues in apoB-100 in LDL during oxidation in the arterial wall could affect the subsequent structural and functional properties.—**Hoff, H. F., and J. O'Neil.** Structural and functional changes in LDL after modification with both 4-hydroxynonenal and malondialdehyde. *J. Lipid Res.* 1993. **34:** 1209–1217.

Supplementary key words oxidized LDL • apoB • aggregation • macrophages • scavenger receptor

LDL accumulation in the arterial intima is considered to be a requisite for the eventual formation of the fatty streak (1), the earliest atherosclerotic lesion (2). It has been suggested that chemical modification of LDL such as oxidation in the intima leads to enhanced uptake by tissue macrophages (3–5), eventually resulting in foam cell formation, the hallmark of the fatty streak lesion (2). One mechanism for such enhanced uptake is recognition by the scavenger receptor on macrophages (5) which, in contrast to the LDL receptor (6), is not down-regulated by intracellular cholesterol content. One chemical modification of LDL that leads to scavenger receptor recognition is interaction with malondialdehyde (MDA), which blocks lysine residues on apoB in LDL by forming a Schiff-base adduct (7). Such blockage first reduces recognition of LDL by the LDL receptor on monocyte-macrophages, and when about 15–20% of exposed lysine residues on apoB in LDL becomes blocked, recognition by the scavenger receptor begins (7). Recently, we showed that when LDL was modified with another aldehyde, 4-hydroxynonenal (HNE) at low LDL concentrations, it also became recognized by the scavenger receptor on macrophages (8). Others have shown that modification of LDL

Abbreviations: LDL, low density lipoproteins; MDA, malondialdehyde; HNE, 4-hydroxynonenal; apoB, apolipoprotein B; ox-LDL, oxidized LDL; ACAT, acyl-CoA: cholesterol acyltransferase; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; BCA, bicinchoninic acid; REM, relative electrophoretic mobility; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TNBS, 2,4,6-trinitrobenzene-1-sulfonic acid; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; TCA, trichloracetic acid; TBARS, thiobarbituric acid-reactive substances.

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with a variety of other aldehydes leads to scavenger receptor recognition on macrophages (3, 9).

One of the characteristics of oxidized LDL is the production of such reactive aldehydes as MDA and HNE by the decomposition of lipid hydroperoxides (5, 9, 10). That both MDA and HNE become incorporated into the oxidized (ox-) LDL particle by covalently coupling to amino acid residues on apoB-100 is suggested by the result that monoclonal antibodies directed at LDL modified with MDA or with HNE cross-react with oxidized LDL (11). That one of the adducts formed between apoB-100 and HNE in ox-LDL is a Schiff-base is suggested from the similarity in fluorescence at 360 ex/430 em between HNE-modified LDL and Cu^{2+}-ox-LDL (8, 10, 12). Thus, it is conceivable that LDL modified with both HNE and MDA might mimic ox-LDL in several structural and functional properties. Recently, we and others have shown that an LDL-sized fraction isolated from human atherosclerotic lesions demonstrates several characteristics of ox-LDL, primary of which are the reactivities with anti-MDA-LDL (13, 14), anti-HNE-LDL (14), and fluorescence properties characteristic of HNE-modified LDL (10). Thus, lesion-derived LDL appears to be modified by both HNE and MDA.

Based on these considerations, we deemed it of importance to characterize the structural and functional properties of LDL modified by both HNE and MDA and compare them to those of LDL modified by HNE alone or MDA alone. As both aldehydes interact with exposed lysine residues on apoB (7, 8, 10), we wished to determine whether modification with both aldehydes would be greater in terms of such blockage of lysine residues and the resultant increases in electrophoretic mobility (8). As modification of LDL at high concentrations (500 μg protein/ml) with sufficient amounts of HNE (5 mM and above) results in particle aggregation (15), we also asked whether co-modification of LDL with HNE and MDA prevented particle aggregation. Finally, we asked whether LDL modified with both aldehydes resulted in additive uptake, degradation, and ACAT stimulation by J774 macrophages relative to LDL modified individually with HNE and with MDA, and whether this uptake was mediated by the scavenger receptor.

MATERIALS AND METHODS

4-Hydroxy-2,3-*trans*-nonenal was synthesized as previously described (12) and was stored as a 10–20 mg/ml solution in dichloromethane at −70°C. Dulbecco's Modified Eagle's Medium (DMEM), fetal calf serum, and penicillin-streptomycin solution were obtained from GIBCO Laboratories (Grand Island, NY). Bovine serum albumin (BSA, fatty acid-free), oleic acid, cholesteryl oleate, and fucoidin were purchased from Sigma Chemical Corporation (St. Louis, MO). The bicinchoninic acid (BCA) protein assay kit was purchased from Pierce Chemicals (Rockford, IL). Premade agarose gels and Fat Red 7B were obtained from Corning (Palto Alto, CA). The cholesterol Reagent Set kit was purchased from Boehringer-Mannheim (Indianapolis, IND). SG-60 plates for thin-layer chromatography were obtained from Merck (Darmstadt, Germany). Carrier-free $Na^{125}I$ was purchased from ICN Pharmaceuticals, Incorporated (Irvine, CA), and [1-^{14}C]oleic acid (48 mCi/mmol) and [1,2,6,7-^{3}H(N)]cholesteryl oleate (82.7 Ci/mmol)] were purchased from New England Nuclear (Wellington, DE).

Preparation of low density lipoproteins and modified forms

Human LDL (d 1.019–1.063 g/ml) was prepared by the procedure of Hatch and Lees (16) from plasma obtained from the CCF Blood Bank. It was stored in 0.15 M NaCl containing 0.3 mM ethylenediaminetetraacetic acid (EDTA), pH 8.5. Acetyl LDL was formed by repeated additions of acetic anhydride to LDL until over 80% of the lysyl residues on apoB were blocked (17). HNE-modified LDL was formed by incubating LDL in the dark under N_2 with an aqueous solution of HNE at final concentrations ranging from 1 to 8 mM in 0.1 M Na phosphate buffer (pH 6.4 or 7.4) containing 0.15 M NaCl and 0.3 mM EDTA for 5 h at 37°C as previously described (15). The HNE-modified LDL was then dialyzed for 12 h against 0.01 M phosphate buffer (pH 7.4), 0.15 M NaCl, and 0.3 mM EDTA to remove unreacted HNE (15). MDA was prepared as described previously (7) and used to modify LDL either at pH 6.4 or pH 7.4. HNE concentrations were determined by spectroscopy by using ultraviolet absorbance at 223 nm and a molar absorption coefficient of 13.75×10^3 (18). MDA concentrations were determined by spectroscopy at 267 nm with a molar absorption coefficient of 3.42×10^4 (19). LDL was modified with mixtures of HNE and MDA at concentrations and pH conditions as stated, using conditions as just described for modifications with HNE. In select experiments LDL was modified sequentially with MDA and with HNE at the pH conditions stated. In these studies LDL was first modified with the one aldehyde, subjected to extensive dialysis, and then modified with the second aldehyde as described above for each aldehyde individually.

LDL was labeled with ^{125}I to a specific activity of 100 cpm/ng protein by using the iodine monochloride procedure described by Bilheimer, Eisenberg, and Levy (20). Acetylation was performed before iodination, whereas modification with HNE or MDA was performed after iodination. The protein content of lipoproteins was determined by the bicinchoninic acid (BCA) assay as described (21) except that a 60-min, 60°C heating step was used, and BSA was used as a standard. The cholesterol content

was determined by a modification of the procedure of Roeschlau, Bernt, and Gruber (22) by using the Reagent Set kit from Boehringer-Mannheim (Indianapolis, IN). Lipoprotein concentrations were routinely expressed as μg protein/ml.

Agarose gel electrophoresis of LDL was performed on pre-made agarose gels according to the manufacturer's instructions at 90 V for 50 min. LDL migration was assigned a value of one, and all other preparations were compared to LDL to obtain a relative electrophoretic mobility (REM). Lipoproteins were visualized by staining with Fat Red 7B in methanol. Vertical slab sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) with a 3–12% polyacrylamide gradient was performed on ^{125}I-labeled lipoproteins according to modifications of the procedure of Laemmli (23). Briefly, samples were reduced and denatured in the presence of SDS and mercaptoethanol as described recently (8), except that samples were heated for 2 min at 100°C and then incubated for 1 h at 55°C. After electrophoresis, the gel was placed in 50% methanol overnight. Wet gels were then sealed in plastic bags and subjected to autoradiography with X-omat K film in an X-matic cassette containing an intensifying screen (Kodak, Rochester, NY).

Fluorescence of modified forms of LDL was determined at 360 nm wavelength excitation and 430 nm wavelength emission by using a Perkin-Elmer LS-3 fluorescence spectrometer (Perkin-Elmer, Norwich, CT) according to a procedure previously described by us (8). Blockage of lysyl residues of the apolipoprotein by HNE, by acetylation, or by MDA was determined by measuring reactivity with 2,4,6-trinitrobenzene-1-sulfonic acid (TNBS) (24), using α-amino-blocked lysine as a standard. SDS (final concentration 1%) was added to the reaction mixture after the reaction was completed but prior to spectroscopy. The amount of MDA bound to apoB in LDL was estimated by the TBARS reaction (25). Solubility of ^{125}I-labeled LDL modified with HNE alone, MDA alone, or mixtures of the two aldehydes, as described above, was determined by measuring the percent of total label still present in the supernatant after centrifugation at 10,000 *g* as previously reported (8).

Uptake of lipoproteins by cells in culture

Macrophage preparation. The J774 macrophage cell line was plated at 5×10^5 cells/16-mm diameter wells. Cells were incubated at 37°C in 5% CO_2 for 48 h in a mixture of DMEM and Ham's F12 media (1:1) containing 5% fetal calf serum as previously reported (15).

Cholesterol esterification. Incorporation of [^{14}C]oleate into cholesteryl oleate was determined by the following modifications of published procedures (17, 25). Cells were rinsed with DMEM and then incubated with [^{14}C]oleate/albumin (final oleate concentration, 0.27 mM) containing the indicated concentration of lipoprotein in DMEM. At 16 h, the cells were rinsed twice with phosphate-buffered saline (PBS). After the final aspiration, 0.5 ml of PBS was added to each well, the cells were scraped using a rubber policeman, and the suspended cells were transferred to a test tube. An aliquot of cell suspension was taken for the determination of total cellular protein. The remaining suspended cells were disrupted with a probe sonicator (Ultrasonics, Incorporated Model W220F, Plainview, NY) at 20 W for 10 sec. The lipids in the sonicated mixture were extracted with hexane-isopropanol 3:2 (v/v) as reported previously (26) after the addition of [^{3}H]cholesteryl oleate as an internal standard. The organic phase was dried under a stream of N_2, and the lipids were separated by thin-layer chromatography in a developing solvent of hexanes–diethyl ether 7:3 (vol/vol). Cholesteryl esters were identified by co-migration with a cholesteryl oleate standard. Radioactivity was quantitated by liquid scintillation counting.

Degradation of ^{125}I-labeled low density lipoprotein. The uptake and subsequent degradation of ^{125}I-labeled LDL by macrophages was measured as described by Goldstein et al. (17) and Morton, West, and Hoff (26) but with some modifications. The cells were incubated with ^{125}I-labeled lipoproteins in DMEM medium containing 6 mg/ml BSA. The extent of degradation was determined as the TCA-soluble radioactivity minus the nonorganic iodine activity. The radioactivity in the cell-free control wells was routinely determined for each condition and was subtracted from that obtained in the presence of cells. Degradation was expressed as μg protein degraded per mg cell protein per time of the incubation period. Lipoprotein concentrations were expressed as μg protein/ml.

RESULTS

Structural properties of LDL modified with MDA and HNE

We had shown previously that when LDL at 500 μg protein/ml was modified with HNE, a dose-dependent increase in particle aggregation was found, estimated by the percent of ^{125}I-labeled LDL that could be precipitated by centrifugation at 10,000 *g* (15). To assess whether modification of LDL with both HNE and MDA resulted in less aggregation, e.g., greater solubility, we compared the solubility of ^{125}I-labeled LDL modified at pH 7.4 concurrently with 5 mM HNE and with increasing amounts of MDA. Even though only 20% of the LDL particles remained soluble after modification with 5 mM HNE, solubility was 60% when LDL was modified with both 5 mM HNE and 10 mM MDA, and 95% when modified with 5 mM HNE and 20 mM MDA (**Fig. 1**). Thus, the presence of MDA prevented aggregation induced by HNE alone. In a separate experiment, LDL was first incubated with 5 mM HNE which resulted in most of the

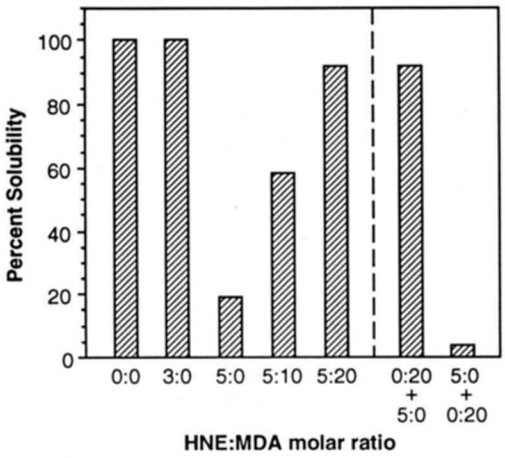

Fig. 1. Dependence of LDL solubility on the concentrations of MDA and HNE used to modify LDL. A sample of ^{125}I-labeled LDL (500 μg/ml) was modified concurrently at pH 7.4 with HNE and MDA at the indicated concentrations of each aldehyde (mM HNE:mM MDA) as described under Materials and Methods. Solubility was estimated as the percent of label not precipitated by centrifugation at 10,000 *g*. ^{125}I-labeled LDL (500 μg/ml) was also modified sequentially with 20 mM MDA, dialyzed, and then modified with 5 mM HNE (0:20 + 5:0), or first with 5 mM HNE and then with 20 mM MDA (5:0 + 0:20). LDL solubility was then determined as just described.

particles aggregating. When this HNE-modified LDL was then dialyzed to remove all unbound HNE and was then incubated with 20 mM MDA, particle aggregation remained the same, suggesting that aggregation was an irreversible process (Fig. 1). However, when LDL was first modified with 20 mM MDA, dialyzed, and then modified with 5 mM HNE, almost no aggregation took place. Thus, the MDA-modified LDL was refractory to aggregation by HNE.

One way by which MDA could prevent the HNE-induced aggregation of LDL (15) would be by preventing intermolecular cross-bridging of apoB in separate particles (8–15). To assess this we subjected ^{125}I-labeled LDL modified at pH 7.4 with 6 mM HNE (HNE-LDL) or with 6 mM HNE and 20 mM MDA (HNE-MDA-LDL) to SDS-PAGE using a 3–12% polyacrylamide gradient followed by autoradiography, and compared the migration of apoB in each sample (**Fig. 2**). The increased solubility of LDL modified concurrently with both aldehydes was also reflected by a greater percent of the total applied label entering into the gel, e.g., 53% of the label applied was still in the stacking gel for HNE-LDL, whereas only 5% was present in the stacking gel for HNE-MDA-LDL. We are assuming that the amount of label remaining in the stacking gel is a measure of the amount of apoB that could not be dissociated by SDS, possibly because of covalent cross-bridging. Furthermore, less of the total labeled applied was present in the B-100 band for HNE-LDL, e.g., 28%, than for HNE-MDA-LDL, e.g., 53%. We also found that 42% of the total applied label for HNE-MDA-LDL, but only 19% for HNE-LDL, was also present in a band (doublet for HNE-MDA-LDL) representing apoB of higher molecular weight than apoB-100. This band may represent intermolecular cross-bridging of individual LDL particles to form dimers, and/or intramolecular cross-bridging affecting migration of apoB in polyacrylamide. Thus, it is likely that MDA prevents particle aggregation induced by HNE, by limiting or reducing inter- and/or intramolecular cross-bridging in LDL by HNE.

To assess the degree of chemical modification of LDL modified by combinations of MDA and HNE at pH 7.4 or with each individually, we measured the REM of each sample of modified LDL on 1% agarose. When LDL was modified with 6 mM HNE and increasing amounts of MDA or with increasing amounts of MDA alone, concentration (MDA)-dependent increases in REM were obtained (**Fig. 3**). Measurement of REM for the only insoluble sample applied, e.g., LDL modified with 6 mM HNE alone, was difficult, as only a small amount of LDL migrated from the origin and the band was diffuse. If this material represented smaller aggregates of LDL, its measured REM might be an underestimate, e.g., its REM might have been 2.5 instead of 2.0 if movement through 1% agarose had not been impeded. This would have given a linear slope parallel with that of MDA alone, suggesting that chemical modification of LDL with HNE and MDA

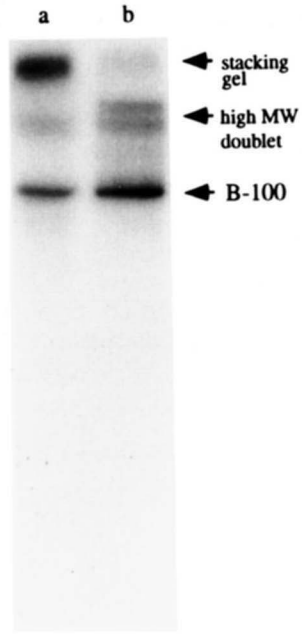

Fig. 2. Autoradiogram of SDS-PAGE of ^{125}I-labeled LDL modified with both MDA and HNE and with HNE alone. ^{125}I-labeled LDL (500 μg/ml) was modified at pH 7.4 with 6 mM HNE alone (a) or with 6 mM HNE plus 20 mM MDA (b), subjected to SDS-PAGE in a 3–12% polyacrylamide gel, and subsequently to autoradiography as described under Materials and Methods. Arrows indicate positions of 3% stacking gel, B-100 band, and a high molecular weight doublet.

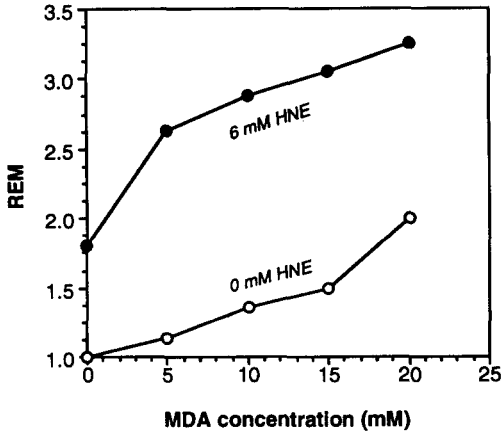

Fig. 3. Effect of concentrations of HNE and MDA added concurrently to LDL on its electrophoretic mobility. LDL (500 μg/ml) was modified with 0 (○) and 6 mM HNE (●) and increasing amounts of MDA at pH 7.4 as described under Materials and Methods. Each sample was then subjected to electrophoresis in 1% agarose and its mobility was measured relative to unmodified LDL (REM).

concurrently was additive. This additive effect was confirmed when, in a separate experiment, we found an approximately additive effect of blockage of exposed lysine residues and REM in LDL modified at pH 7.4 with 6 mM HNE alone plus with 20 mM MDA alone, compared with LDL modified with both 6 mM HNE and 20 mM MDA (**Table 1**). We could show that such blockage of lysine residues on apoB in LDL modified at pH 7.4 with MDA was due directly to the binding of MDA to apoB. Values obtained by the TNBS assay corresponded well with values of blocked lysines obtained by measuring bound MDA with the TBARS assay by assuming that each MDA molecule binds two adjacent lysine residues on apoB (**Table 2**), as suggested previously (7).

TABLE 1. Association of blockage of lysine residues and REM for LDL modified concurrently with MDA and HNE

	HNE Added			
	None		6 mM	
MDA Added	% Blocked Lysines	REM	% Blocked Lysines	REM
mM				
0	0	1.0	30	1.8
20	31	2.4	56	3.1

LDL (500 μg/ml) was modified at pH 7.4 with the indicated concentrations of MDA and HNE as described in Material and Methods. Each sample was then subjected to the TNBS assay to determine blockage of exposed lysine residues on apoB of LDL and to 1% agarose electrophoresis to determine the REM of each modified LDL. When LDL was modified with 6 mM HNE alone, about 90% of the particles were insoluble. However, a small percent did migrate from the origin during electrophoresis, and its REM could be measured.

TABLE 2. MDA bound to apoB and blockage of lysine residues on apoB in LDL after modification with MDA at pH 7.4

MDA Concentration Added to LDL	mol MDA Bound per mol ApoB	% Blocked by Lysines	
		Calculated from TBARS	Determined by TNBS
5	38	21	16
10	48	26	20
15	51	28	26
20	58	32	33

LDL (500 μg/ml) was modified with the indicated concentrations of MDA at pH 7.4. Each sample was then subjected to the TNBS reaction to determine the % of blocked lysines and to the TBARS reaction to determine the number of MDA molecules bound to each molecule of apoB. This number was converted to % of blocked lysines, assuming that two lysines on apoB were blocked by each bound MDA molecule, as suggested previously (7).

Functional properties of LDL modified with HNE and MDA

To determine whether modification of LDL with both aldehydes affected its uptake and degradation by the J774 macrophage cell line, we incubated these cells with ^{125}I-labeled LDL modified at pH 7.4 with 8 mM HNE or 0 mM HNE (sham treatment) and increasing amounts of MDA up to 40 mM and determined the amount of degradation. ^{125}I-labeled LDL modified with 8 mM HNE alone or with 8 mM HNE and increasing amounts of MDA showed an essentially linear increase in degradation with increasing modifications with MDA (**Fig. 4a**). In

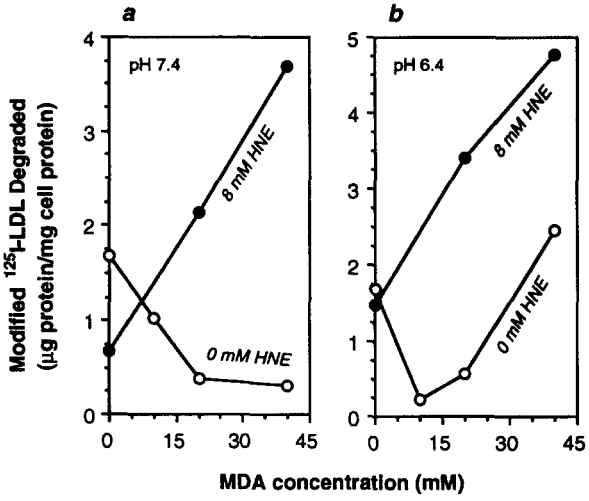

Fig. 4. Effect of modification of ^{125}I-labeled LDL with HNE and MDA at pH 7.4 and at pH 6.4 on its degradation by J774 macrophages. ^{125}I-labeled LDL was modified concurrently at pH 7.4 (Fig. 4a) or at pH 6.4 (Fig. 4b) with 0 mM HNE (○) or 8 mM HNE (●) and increasing amounts of MDA as described under Materials and Methods and incubated with J774 macrophages at a final concentration of 20 μg/ml in incubation medium for 4.5 h at 37°C. Lipoprotein degradation was determined as described under Materials and Methods. Data points represent the mean of duplicate determinations.

LDL modified with 8 mM HNE alone at pH 7.4, 94% of the particles were aggregated. LDL modified with 8 mM MDA at pH 7.4 was completely soluble (not shown). As the J774 cells used in this study failed to phagocytose aggregated forms of LDL (not shown), we were presumably measuring only the contribution of the 6% of the LDL particles still soluble following modification with 8 mM HNE. Degradation of ^{125}I-labeled LDL modified with 8 mM HNE and with 20 or 40 mM MDA was found to be more than the sum of degradation of ^{125}I-labeled LDL modified with 8 mM HNE alone and ^{125}I-labeled LDL modified with 20 or 40 mM MDA alone. LDL modified only with MDA showed an MDA-dependent reduction in degradation.

Because in previous studies modification of LDL with MDA was performed at pH 6.4 (7) rather than the pH 7.4 used in our studies, we asked whether blockage of exposed lysine residues on apoB-100 and REM of LDL would differ between LDL modified with increasing amounts of MDA at pH 7.4 and at pH 6.4. We, therefore, plotted J774 macrophage degradation against increases of either REM or of a blockage of lysines residues in nine sets of LDL samples modified at either pH 6.4 or pH 7.4 with increasing amounts of MDA up to 50 mM (**Fig. 5**). It can be readily seen that REM and % lysines blocked showed a linear relationship, and both were higher for LDL modified at pH 6.4 than at 7.4. LDL modified with the same concentration of MDA is given the same number. An initial reduction was found in macrophage recognition for LDL modified with low concentrations of MDA at pH 6.4 or at pH 7.4 due to a decreased recognition by the LDL receptor, as indicated by competition studies with excess LDL (not shown). At higher MDA concentrations we found a concentration-dependent increase in macrophage degradation for both LDL modified at pH 6.4 or at pH 7.4. This increase was the result of enhanced recognition by the scavenger receptor, based on competition studies with excess acetyl LDL (not shown). As had been reported previously (7), we found that this increase in degradation occurred when 15–20% of the lysines in apoB were blocked by MDA, and that degradation reached a plateau for LDL modified at higher MDA concentrations at pH 6.4. However, not shown previously was the observation that even at equivalent degrees of modification in terms of blockage of lysine residues or REM, LDL modified at pH 6.4 was degraded better than when modified at pH 7.4 (Fig. 5). This result suggests that additional modifications of LDL by MDA that occur at pH 6.4 but not at pH 7.4 result in further enhancement of scavenger recognition. It should be noted that the increase in macrophage degradation seen in Fig. 5, for LDL modified with MDA at pH 7.4 as contrasted to the steady decrease seen in Fig. 4a, is due to the greater degree of modification of LDL with MDA in Fig. 5.

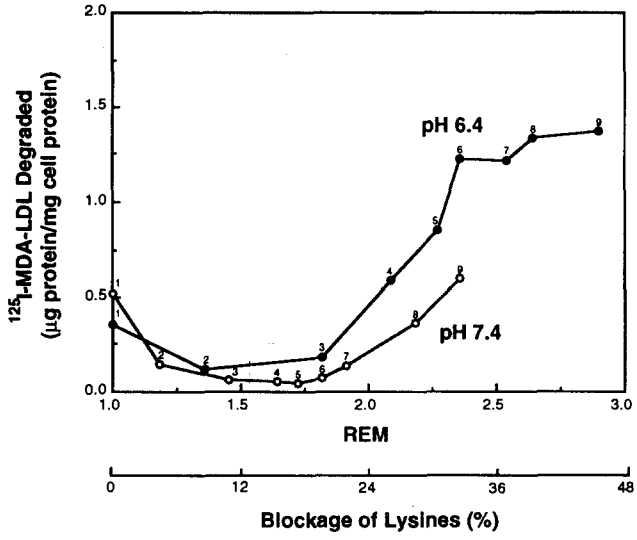

Fig. 5. Macrophage degradation of LDL modified by MDA at pH 6.4 or at pH 7.4 to equal degrees as estimated by particle REM and blockage of lysine residues. ^{125}I-labeled LDL (500 μg/ml) was modified with MDA at either pH 6.4 or pH 7.4 at concentrations ranging from 0 to 50 mM. This resulted in increases in particle REM ranging from 1 to 3 that corresponded to blockage of lysine residues ranging from 0 to 48%. Each individual sample of MDA-modified ^{125}I-labeled LDL was incubated for 4.5 h at 37°C with J774 macrophages at a final concentration of 20 μg protein/ml and degradation was determined as described under Materials and Methods. The same number of samples were modified at pH 6.4 and pH 7.4. Sets of the nine LDL samples modified by increasing concentrations of MDA but at pH 6.4 or pH 7.4, are identified by individual numbers. Data points represent the mean of triplicate determinations.

When LDL was modified with HNE and MDA at pH 6.4 and then incubated with J774 macrophages, degradation of ^{125}I-labeled LDL modified by both aldehydes showed a linear increase with amount of MDA added (Fig. 4b) which was slightly greater than corresponding values for LDL modified at pH 7.4 (Fig. 4a). Degradation of LDL modified with 8 mM HNE at pH 6.4 was somewhat higher than degradation of LDL when modified at pH 7.4, possibly due to the fact that in the former only 63% of the particles were aggregated as contrasted to 94% in the latter. LDL modified with only MDA showed the same initial reduction and subsequent increase in degradation with increasing MDA added, as seen in Fig. 5.

To assess whether this degradation in J774 macrophages also resulted in subsequent stimulation of cholesterol esterification, we asked whether unlabeled LDL modified at pH 7.4 with 6 mM HNE and increasing amounts of MDA also showed the MDA concentration-dependent increase in stimulation of cholesterol esterification as just reported for degradation. When LDL modified at pH 7.4 with 6 mM HNE or 0 mM HNE and increasing amounts of MDA, was incubated with J774 macrophages for 16 h, stimulation of cholesterol esterification (**Fig. 6**) indicating stimulation of ACAT (6) paralleled the degradation rates obtained in a separate experiment (Fig. 4a). Similar results were obtained for stimulation of cho-

lesterol esterification for MDA modified with both aldehydes at pH 6.4 (not shown).

To determine whether the scavenger receptor was responsible for the binding and uptake of LDL modified with both HNE and MDA, we performed cross-competition studies with acetyl LDL and with LDL modified at pH 7.4 with 6 mM HNE and 40 mM MDA. As seen in **Fig. 7**, excess acetyl LDL was at least as effective in inhibiting the degradation of ^{125}I-labeled HNE-MDA-LDL as of ^{125}I-labeled acetyl LDL. Excess HNE-MDA-LDL was able to quantitatively inhibit the degradation of both ^{125}I-labeled HNE-MDA-LDL and ^{125}I-labeled acetyl LDL, but with an apparently lower affinity than acetyl LDL, based on initial slopes of these competition curves. These data suggest that the degradation of ^{125}I-labeled HNE-MDA in J774 macrophages is mediated by the scavenger receptor on these cells. Since fucoidin is also a ligand for the scavenger receptor (27), we also asked whether fucoidin would inhibit the stimulation of cholesterol esterification in J774 macrophages induced by HNE-MDA-LDL. When LDL, modified with 6 mM HNE and 40 mM MDA, was incubated for 16 h at 37°C with macrophages in the presence and absence of 100 μg/ml fucoidin, we found that fucoidin inhibited esterification by 85%, e.g., from 34.5 nmol to 5.5 nmol cholesterol esterified/mg cell protein.

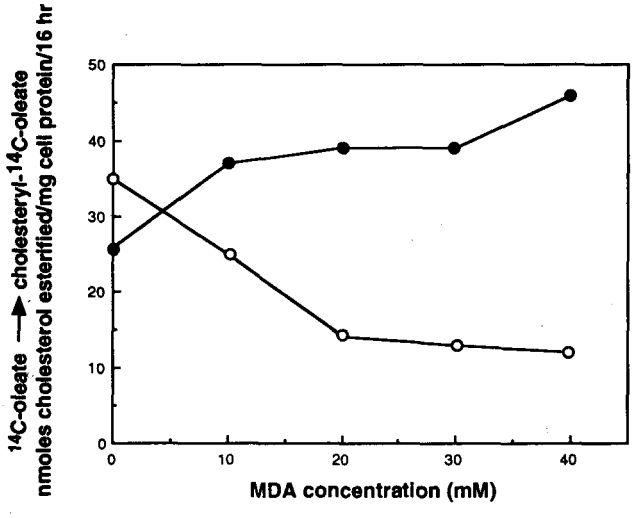

Fig. 6. Stimulation of cholesterol esterification in J774 macrophages by LDL modified concurrently with mixtures of HNE and MDA. LDL modified concurrently at pH 7.4 with 0 mM HNE (○), or 8 mM HNE (●) and increasing concentrations of MDA as indicated, were incubated for 16 h at 37°C at a final concentration of 100 μg cholesterol/ml with J774 macrophages in medium containing [^{14}C]oleate-albumin. The amount of cholesteryl [^{14}C]oleate formed was quantified as described under Materials and Methods. Data points represent the mean of triplicate determinations.

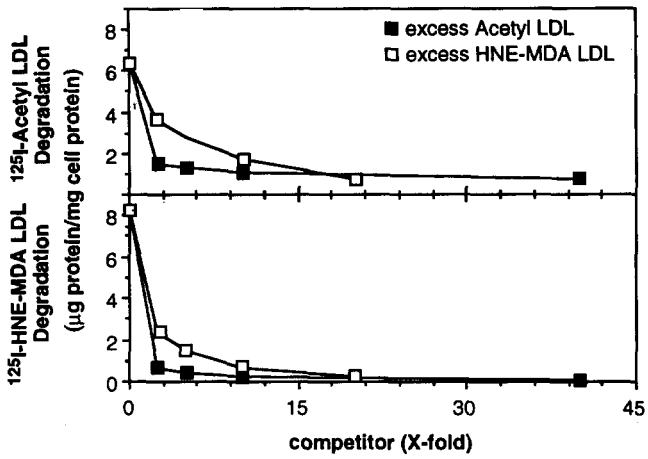

Fig. 7. Inhibition by unlabeled acetyl LDL and HNE-MDA-modified LDL of the degradation in J774 macrophages of ^{125}I-labeled acetyl LDL and ^{125}I-labeled HNE-MDA-modified LDL. ^{125}I-labeled acetyl LDL and ^{125}I-labeled LDL (500 μg/ml) modified concurrently with 6 mM HNE and 40 mM MDA at pH 7.4 were incubated with J774 macrophages, each at a final concentration of 20 μg/ml in incubation medium in the presence of increasing amounts of unlabeled acetyl LDL (■) or unlabeled LDL modified with 6 mM HNE and 40 mM MDA (□) as indicated. Lipoprotein degradation was determined as described under Materials and Methods. Data points represent the mean of duplicate determinations.

DISCUSSION

The results from this study indicate that modification of LDL with two major aldehydes formed during lipid peroxidation, the bifunctional HNE and the hydrophilic MDA, results in significant changes in structural and functional properties of LDL. Simultaneous modification of LDL with both aldehydes (or pretreatment of LDL with MDA prior to modification with HNE) prevented particle aggregation induced by modifying LDL with high concentrations of HNE alone. The increased solubility of delipidated apoB in HNE-MDA-modified LDL relative to HNE-modified LDL, as seen in SDS-PAGE gels, suggests that MDA reduces the HNE-induced cross-linking of apoB in separate LDL particles, as suggested previously in HNE-modified LDL (15) and extensively oxidized LDL (8). Modification of LDL with both HNE and MDA at pH 7.4 induced essentially additive increases in REM and blockage of free lysines on apoB over the ranges of aldehyde concentrations studied.

The chemical modification of LDL by both aldehydes also resulted in enhanced uptake and intracellular degradation via the scavenger receptor on J774 macrophages and subsequent stimulation of ACAT. When modifications of LDL with HNE and MDA were performed at pH 6.4, at which reactivity with MDA was shown to be higher than at pH 7.4, macrophage degradation was closer to being additive at higher concentrations of MDA than at pH

7.4 when plotted in terms of MDA concentrations added. One explanation for this observation is that even though the addition of more MDA does not form additional Schiff-base adducts with apoB at pH 7.4, it does prevent aggregation of LDL by HNE. Since we have shown previously that when LDL is modified with HNE alone to sufficiently high levels to induce aggregation, blockage of lysine residues on apoB ceases when extensive aggregation increases (15). It is possible that by preventing aggregation MDA allows HNE to modify more lysine residues, thereby leading to enhanced degradation as a result of enhanced scavenger receptor recognition. That this uptake of HNE-MDA-LDL is mediated by the scavenger receptor was shown by cross-competition studies of degradation with excess acetyl LDL and inhibition of cholesterol esterification with fucoidin. Our data also indicate that at pH 7.4 such doubly modified LDL could induce lipid loading in tissue macrophages, as indicated by the stimulation of cholesterol esterification. Whether our data obtained for LDL modified in vitro with HNE and MDA at pH 6.4 has any physiologic relevance depends on whether the interstitial space in regions of lysed cells could reach a pH of 6.4. It is of note that when LDL was modified with MDA at pH 6.4, the resulting adduct was degraded by macrophages to a greater extent than LDL modified with MDA at pH 7.4, even at equivalent degrees of modification as assessed by REM or blockage of lysine residues. This suggests that either additional MDA is bound to LDL at pH 6.4 that is not bound as Schiff bases to lysine residues, or the interaction of apoB with MDA at pH 6.4 induces conformational changes that somehow leads to enhanced scavenger receptor recognition. Further studies are needed to better clarify these mechanisms.

Although oxidation was identified in LDL-sized particles extracted from lesions (13, 14), it is possible that larger sized particles may be also present that represent aggregated forms of ox-LDL. This is, in part, based on the observation that both lesion-derived LDL and moderately ox-LDL underwent aggregation when concentrated to levels of over 1 mg/ml (13). Likewise, LDL subjected to Cu^{2+}-induced oxidation demonstrated extensive particle aggregation when oxidized at 500 μg/ml (8). As this concentration of LDL was shown to be present in grossly normal intima and in plaques of humans (28, 29), it is conceivable that aggregated forms of ox-LDL are present in lesions. The prevention of aggregation of concentrated forms of LDL induced by HNE alone, when also modified in the presence of another aldehyde such as MDA, suggests that MDA released during lipid peroxidation can play a major role in altering the structural and functional properties of LDL when co-modified with other aldehydes such as HNE. Aggregated forms of modified LDL become internalized by a scavenger receptor-independent route involving phagocytosis (13, 15, 30), whereas, as shown in this study, soluble forms of LDL modified by both HNE and MDA demonstrate receptor-mediated endocytosis via the scavenger receptor. Perhaps there are specific situations in which oxidation results in much more HNE being created than MDA, resulting in increased particle aggregation. Because of its hydrophilic properties, MDA can diffuse away from the ox-LDL particle more readily than HNE (10). If conditions in the extracellular milieu of the arterial intima accelerate or impede such diffusion of MDA, they could determine whether LDL undergoing oxidation in the vessel wall becomes aggregated or not. Clearly, further work is needed to assess what factors control the formation and interactions of reactive aldehydes with neighboring proteins during LDL oxidation in the arterial wall.

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